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Osteogenic Protein2-7-92  
Linda B.Reference to Related Applications

This application is a continuation-in-part of copending U.S. Application Serial No. 569,920, (filed August 20, 1990 and entitled "Osteogenic Devices"), and U.S. Application Serial No. 422,699, (filed October 17, 1989 and entitled "Osteogenic Protein"), which are both continuation-in-parts of U.S. Application Serial No. 315,342, filed February 23, 1989, and entitled "Osteogenic Devices."

Background of the Invention

This invention relates to novel polypeptide chains and to osteogenic proteins comprising these polypeptide chains which are capable of inducing osteogenesis in mammals; to genes encoding the polypeptide chains; to methods for their production using recombinant DNA techniques, and to bone and cartilage repair procedures using the osteogenic proteins.

Mammalian bone tissue is known to contain one or more proteinaceous materials, presumably active during growth and natural bone healing, which can induce a developmental cascade of cellular events resulting in endochondral bone formation. This active factor (or factors) has variously been referred to in the literature as bone morphogenetic

or morphogenic protein, bone inductive protein, osteogenic protein, osteogenin, or osteoinductive protein.

The developmental cascade of bone differentiation consists of recruitment of mesenchymal cells, proliferation of progenitor cells, calcification of cartilage, vascular invasion, bone formation, remodeling, and finally marrow differentiation (Reddi (1981) Collagen Rel. Res. 1:209-226).

Though the precise mechanisms underlying these phenotypic transformations are unclear, it has been shown that the natural endochondral bone differentiation activity of bone matrix can be dissociatively extracted and reconstituted with inactive residual collagenous matrix to restore full bone induction activity (Sampath and Reddi, (1981) Proc. Natl. Acad. Sci. USA 78:7599-7603). This provides an experimental method for assaying protein extracts for their ability to induce endochondral bone in vivo. Several species of mammals produce closely related protein as demonstrated by cross species implant experiments (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591-6595).

The potential utility of these proteins has been recognized widely. It is contemplated that the availability of the protein would revolutionize orthopedic medicine, certain types of plastic surgery, and various periodontal and craniofacial reconstructive procedures.

The observed properties of these protein fractions have induced an intense research effort in various laboratories directed to isolating and identifying the pure factor or factors responsible for osteogenic activity. The current state of the art of purification of osteogenic protein from mammalian bone is disclosed by Sampath et al. ((1987) Proc. Natl. Acad. Sci. USA 80). Urist et al. (1984) Proc. Soc. Exp. Biol. Med. 173:194-199 disclose a human osteogenic protein fraction which was extracted from demineralized cortical bone by means of a calcium chloride-urea inorganic-organic solvent mixture, and retrieved by differential precipitation in guanidine-hydrochloride and preparative gel electrophoresis. The authors report that the protein fraction has an amino acid composition of an acidic polypeptide and a molecular weight in a range of 17-18 kD.

Urist et al. (1984) Proc. Natl. Acad. Sci. USA 81:371-375 disclose a bovine bone morphogenetic protein extract having the properties of an acidic polypeptide and a molecular weight of approximately 18 kD. The authors reported that the protein was present in a fraction separated by hydroxyapatite chromatography, and that it induced bone formation in mouse hindquarter muscle and bone regeneration in trephine defects in rat and dog skulls. Their method of obtaining the extract from bone results in ill-defined and impure preparations.

European Patent Application Serial No. 148,155, published October 7, 1985, purports to disclose osteogenic proteins derived from bovine,

porcine, and human origin. One of the proteins, designated by the inventors as a P3 protein having a molecular weight of 22-24 kD, is said to have been purified to an essentially homogeneous state. This material is reported to induce bone formation when implanted into animals.

International Application No. PCT/087/01537, published January 14, 1988, discloses an impure fraction from bovine bone which has bone induction qualities. The named applicants also disclose putative "bone inductive factors" produced by recombinant DNA techniques. Four DNA sequences were retrieved from human or bovine genomic or cDNA libraries and expressed in recombinant host cells. While the applicants stated that the expressed proteins may be bone morphogenic proteins, bone induction was not demonstrated, suggesting that the recombinant proteins are not osteogenic. The same group reported subsequently (Science, 242:1528, Dec, 1988) that three of the four factors induce cartilage formation, and postulate that bone formation activity "is due to a mixture of regulatory molecules" and that "bone formation is most likely controlled ... by the interaction of these molecules." Again, no bone induction was attributed to the products of expression of the cDNAs. See also Urist et al., EP 0,212,474 entitled Bone Morphogenic Agents.

Wang et al. (1988) Proc. Nat. Acad. Sci. USA 85: 9484-9488 discloses the purification of a bovine bone morphogenetic protein from guanidine extracts of demineralized bone having cartilage and bone formation activity as a basic protein corresponding

to a molecular weight of 30 kD determined from gel elution. Purification of the protein yielded proteins of 30, 18 and 16 kD which, upon separation, were inactive. In view of this result, the authors acknowledged that the exact identity of the active material had not been determined.

Wang et al. (1990) Proc. Nat. Acad. Sci. USA 87: 2220-2227 describes the expression and partial purification of one of the cDNA sequences described in PCT 87/01537. Consistent cartilage and/or bone formation with their protein requires a minimum of 600 ng of 50% pure material.

International Application No. PCT/89/04458 published April 19, 1990 (Int. Pub. No. WO90/003733), describes the purification and analysis of a family of osteogenic factors called "P3 OF 31-34". The protein family contains at least four proteins, which are characterized by peptide fragment sequences. The impure mixture P3 OF 31-34 is assayed for osteogenic activity. The activity of the individual proteins is neither assessed nor discussed.

It is an object of this invention to provide novel polypeptide chains useful as subunits of dimeric osteogenic proteins capable of endochondral bone formation in allogenic and xenogenic implants in mammals, including humans. Another object is to provide genes encoding these polypeptide chains and methods for the production of osteogenic proteins comprising these polypeptide chains using recombinant DNA techniques.

These and other objects and features of the invention will be apparent from the description, drawings, and claims which follow.

### Summary of the Invention

This invention provides novel polypeptide chains useful as either one or both subunits of dimeric osteogenic proteins which, when implanted in a mammalian body in association with a matrix, can induce at the locus of the implant the full developmental cascade of endochondral bone formation and bone marrow differentiation.

A key to these developments was the elucidation of amino acid sequence and structure data of native bovine osteogenic protein. A protocol was developed which results in retrieval of active, substantially pure osteogenic protein from bovine bone having a half-maximum bone forming activity of about 0.8 to 1.0 ng per mg of implant. The availability of the material enabled the inventors to elucidate key structural details of the protein necessary to achieve bone formation. Knowledge of the protein's amino acid sequence and other structural features enabled the identification and cloning of native genes in the human genome.

Consensus DNA sequences based on partial sequence data and observed homologies with regulatory proteins disclosed in the literature were used as probes for extracting genes encoding osteogenic protein from human genomic and cDNA libraries. One of the consensus sequences was used to isolate a previously unidentified gene which, when expressed, encoded a protein comprising a region capable of inducing endochondral bone formation when properly modified, incorporated in a suitable matrix, and

implanted as disclosed herein. The gene, called OP-1 (human OP-1), is described in greater detail in copending U.S. 422,699, the disclosure of which is herein incorporated by reference.

Fragments of the OP-1 DNA sequence subsequently were used to probe a mouse embryo cDNA library in search of additional genes encoding osteogenic proteins. This process isolated several heretofore unidentified DNA sequences. One DNA sequence identified, referred to herein as mOP-1, is discussed in detail in copending (Attorney docket #CRP-001CP5). Another DNA sequence isolated, referred to herein as mOP-2, has significant homology (74%) with the C-terminal active region of OP-1 that comprises the minimally required sequence for osteogenesis (residues 38-139 of Sequence ID No. 6. See, for example, U.S. Application Serial No. 315,342), but less homology with the intact mature form of OP-1 (about 58%). Moreover, mature mOP-2 has eight cysteine residues in its sequence, as compared with other native osteogenic proteins identified to date, which have only seven cysteines in this region.

The sequence of what is believed to be the mature form of mOP-2 is (Sequence ID No. 1):

mOP-2

Ala	Ala	Arg	Pro	Leu	Lys	Arg	Arg	Gln
1				5				
Pro	Lys	Lys	Thr	Asn	Glu	Leu	Pro	His
10					15			



Pro	Asn	Lys	Leu	Pro	Gly	Ile	Phe	Asp
	20					25		
Asp	Gly	His	Gly	Ser	Arg	Gly	Arg	Glu
		30					35	
Val	Cys	Arg	Arg	His	Glu	Leu	Tyr	Val
			40					45
Arg	Phe	Arg	Asp	Leu	Gly	Trp	Leu	Asp
				50				
Trp	Val	Ile	Ala	Pro	Gln	Gly	Tyr	Ser
55					60			
Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ala
	65					70		
Phe	Pro	Leu	Asp	Ser	Cys	Met	Asn	Ala
		75					80	
Thr	Asn	His	Ala	Ile	Leu	Gln	Ser	Leu
			85					90
Val	His	Leu	Met	Lys	Pro	Asp	Val	Val
				95				
Pro	Lys	Ala	Cys	Cys	Ala	Pro	Thr	Lys
100					105			
Leu	Ser	Ala	Thr	Ser	Val	Leu	Tyr	Tyr
	110					115		
Asp	Ser	Ser	Asn	Asn	Val	Ile	Leu	Arg
		120					125	
Lys	His	Arg	Asn	Met	Val	Val	Lys	Ala
			130					135
Cys	Gly	Cys	His.					

The amino acid sequence of the full length protein (the "prepro" form), and the cDNA sequence encoding it, are set forth in Seq. ID. No. 2.

A DNA hybridization probe, specific to the

N-terminus of the mature form of mOP-2 then was used to probe several human DNA libraries, and identified a novel sequence, herein referred to as hOP-2, which has substantial homology to mOP-2: about 92% homology throughout the mature form sequence, and about 95% homology in the minimally required C-terminal active region, (residues 38-139). Like mOP-2, mature hOP-2 also contains eight cysteine residues, and has less than 60% homology with mature OP-1.

The sequence of what is believed to be the mature form of hOP-2 is (Sequence ID No. 3):

hOP-2

Ala	Val	Arg	Pro	Leu	Arg	Arg	Arg	Gln
1				5				
Pro	Lys	Lys	Ser	Asn	Glu	Leu	Pro	Gln
10					15			
Ala	Asn	Arg	Leu	Pro	Gly	Ile	Phe	Asp
	20					25		
Asp	Val	His	Gly	Ser	His	Gly	Arg	Gln
		30					35	
Val	Cys	Arg	Arg	His	Glu	Leu	Tyr	Val
			40					45
Ser	Phe	Gln	Asp	Leu	Gly	Trp	Leu	Asp
				50				
Trp	Val	Ile	Ala	Pro	Gln	Gly	Tyr	Ser
55					60			
Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ser
	65					70		
Phe	Pro	Leu	Asp	Ser	Cys	Met	Asn	Ala
		75					80	

Thr	Asn	His	Ala	Ile	Leu	Gln	Ser	Leu
			85					90
Val	His	Leu	Met	Lys	Pro	Asn	Ala	Val
				95				
Pro	Lys	Ala	Cys	Cys	Ala	Pro	Thr	Lys
100					105			
Leu	Ser	Ala	Thr	Ser	Val	Leu	Tyr	Tyr
	110					115		
Asp	Ser	Ser	Asn	Asn	Val	Ile	Leu	Arg
		120					125	
Lys	Ala	Arg	Asn	Met	Val	Val	Lys	Ala
			130					135
Cys	Gly	Cys	His.					

Additional mature species thought to be active include two short sequences:

hOP-2P (SEQ ID NO. 9)

			Pro	Leu	Arg	Arg	Arg	Gln
			1				5	
Pro	Lys	Lys	Ser	Asn	Glu	Leu	Pro	Gln
			10					15
Ala	Asn	Arg	Leu	Pro	Gly	Ile	Phe	Asp
				20				
Asp	Val	Asn	Gly	Ser	His	Gly	Arg	Gln
25					30			
Val	Cys	Arg	Arg	His	Glu	Leu	Tyr	Val
	35					40		
Ser	Phe	Gln	Asp	Leu	Gly	Trp	Leu	Asp
		45					50	
Tyr	Val	Ile	Ala	Pro	Gln	Gly	Tyr	Ser
			55					60

Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ser
				65				
Phe	Pro	Leu	Asp	Ser	Cys	Met	Asn	Ala
70					75			
Thr	Asn	His	Ala	Ile	Leu	Gln	Ser	Leu
	80					85		
Val	His	Leu	Met	Lys	Pro	Asn	Ala	Val
		90					95	
Pro	Lys	Ala	Cys	Cys	Ala	Pro	Thr	Lys
			100					105
Leu	Ser	Ala	Thr	Ser	Val	Leu	Tyr	Tyr
				110				
Asp	Glu	Ser	Asn	Asn	Val	Ile	Leu	Arg
115					120			
Lys	Ala	Arg	Asn	Met	Val	Val	Lys	Ala
	125					130		
Cys	Gly	Cys	His;					
		135						

hOP-2R (SEQ ID NO. 10):

						Arg	Arg	Gln
						1		
Pro	Lys	Lys	Ser	Asn	Glu	Leu	Pro	Gln
	5					10		
Ala	Asn	Arg	Leu	Pro	Gly	Ile	Phe	Asp
		15					20	
Asp	Val	Asn	Gly	Ser	His	Gly	Arg	Gln
			25					30
Val	Cys	Arg	Arg	His	Glu	Leu	Tyr	Val
				35				
Ser	Phe	Gln	Asp	Leu	Gly	Trp	Leu	Asp
40					45			

Tyr	Val	Ile	Ala	Pro	Gln	Gly	Tyr	Ser
	50					55		
Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ser
		60					65	
Phe	Pro	Leu	Asp	Ser	Cys	Met	Asn	Ala
			70					75
Thr	Asn	His	Ala	Ile	Leu	Gln	Ser	Leu
				80				
Val	His	Leu	Met	Lys	Pro	Asn	Ala	Val
85					90			
Pro	Lys	Ala	Cys	Cys	Ala	Pro	Thr	Lys
	95					100		
Leu	Ser	Ala	Thr	Ser	Val	Leu	Tyr	Tyr
		105					110	
Asp	Glu	Ser	Asn	Asn	Val	Ile	Leu	Arg
			115					120
Lys	Ala	Arg	Asn	Met	Val	Val	Lys	Ala
				125				
Cys	Gly	Cys	His;					
130								

and a longer sequence:

hOP-2S (SEQ ID NO. 11)

						Ser	Gln	Gln
						1		
Pro	Phe	Val	Val	Thr	Phe	Phe	Arg	Ala
	5					10		
Ser	Pro	Ser	Pro	Ile	Arg	Thr	Pro	Arg
		15					20	
Ala	Val	Arg	Pro	Leu	Arg	Arg	Arg	Gln
			25					30

Pro	Lys	Lys	Ser	Asn	Glu	Leu	Pro	Gln
				35				
Ala	Asn	Arg	Leu	Pro	Gly	Ile	Phe	Asp
40					45			
Asp	Val	Asn	Gly	Ser	His	Gly	Arg	Gln
	50					55		
Val	Cys	Arg	Arg	His	Glu	Leu	Tyr	Val
		60					65	
Ser	Phe	Gln	Asp	Leu	Gly	Trp	Leu	Asp
			70					75
Tyr	Val	Ile	Ala	Pro	Gln	Gly	Tyr	Ser
				80				
Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ser
85					90			
Phe	Pro	Leu	Asp	Ser	Cys	Met	Asn	Ala
	95					100		
Thr	Asn	His	Ala	Ile	Leu	Gln	Ser	Leu
		105					110	
Val	His	Leu	Met	Lys	Pro	Asn	Ala	Val
			115					120
Pro	Lys	Ala	Cys	Cys	Ala	Pro	Thr	Lys
				125				
Leu	Ser	Ala	Thr	Ser	Val	Leu	Tyr	Tyr
130					135			
Asp	Glu	Ser	Asn	Asn	Val	Ile	Leu	Arg
	140					145		
Lys	Ala	Arg	Asn	Met	Val	Val	Lys	Ala
		150					165	
Cys	Gly	Cys	His.					
			170					

The amino acid sequence of the full length hOP-2 protein (the "prepro" form), and the cDNA sequence encoding it, are set forth in Seq. ID. No. 4.

The amino acid sequences for the mature OP-1, mOP-1, mOP-2 and hOP-2 polypeptide chains are compared in Figure 2. All four amino acid sequences show significant homology in the mature form. Thus, in one preferred aspect, the invention comprises osteogenic proteins comprising a species of polypeptide chain having an amino acid sequence sufficiently duplicative of the sequence of Sequence ID No. 1 or Sequence ID No. 3 such that a dimeric protein comprising this polypeptide chain has a conformation capable of inducing endochondral bone formation when implanted in a mammal in association with a matrix.

In another preferred aspect, the invention comprises osteogenic proteins comprising species of polypeptide chains having the generic amino acid sequence below which accomodates the homologies between these four proteins (sequence ID No. 5):

Cys	Xaa <sub>1</sub>	Xaa <sub>2</sub>	His	Glu	Leu	Tyr	Val	Xaa <sub>3</sub>	Phe	
1				5					10	
Xaa <sub>4</sub>	Asp	Leu	Gly	Trp	Xaa <sub>5</sub>	Asp	Trp	Xaa <sub>6</sub>	Ile	
				15					20	
Ala	Pro	Xaa <sub>7</sub>	Gly	Tyr	Xaa <sub>8</sub>	Ala	Tyr	Tyr	Cys	
				25					30	
Glu	Gly	Cys	Xaa <sub>9</sub>	Phe	Pro	Leu	Xaa <sub>10</sub>	Ser	Xaa <sub>11</sub>	
				35					40	
Met	Asn	Ala	Thr	Asn	His	Ala	Ile	Xaa <sub>12</sub>	Thr	
				45					50	
Leu	Xaa <sub>13</sub>	Xaa <sub>14</sub>	Xaa <sub>15</sub>	Xaa <sub>16</sub>	Xaa <sub>17</sub>	Xaa <sub>18</sub>	Val			
				55						
Pro	Lys	Xaa <sub>19</sub>	Cys	Cys	Ala	Pro	Thr	Xaa <sub>20</sub>	Leu	
		60						65		

Xaa <sub>21</sub>	Ala	Xaa <sub>22</sub>	Ser	Val	Leu	Tyr	Xaa <sub>23</sub>	Asp
	70						75	
Xaa <sub>24</sub>	Ser	Xaa <sub>25</sub>	Asn	Val	Xaa <sub>26</sub>	Leu	Xaa <sub>27</sub>	Lys
		80					85	
Xaa <sub>28</sub>	Pro	Asn	Met	Val	Val	Xaa <sub>29</sub>	Ala	Cys Gly
			90					95

Cys His,

wherein Xaa<sub>1</sub> = (Lys or Arg); Xaa<sub>2</sub> = (Lys or Arg);  
 Xaa<sub>3</sub> = (Ser or Arg); Xaa<sub>4</sub> = (Arg or Gln); Xaa<sub>5</sub> = (Gln  
 or Leu); Xaa<sub>6</sub> = (Ile or Val); Xaa<sub>7</sub> = (Glu or Gln);  
 Xaa<sub>8</sub> = (Ala or Ser); Xaa<sub>9</sub> = (Ala or Ser); Xaa<sub>10</sub> =  
 (Asn or Asp); Xaa<sub>11</sub> = (Tyr or Cys); Xaa<sub>12</sub> = (Val or  
 Leu); Xaa<sub>13</sub> = (His or Asn); Xaa<sub>14</sub> = (Phe or Leu);  
 Xaa<sub>15</sub> = (Ile or Met); Xaa<sub>16</sub> = (Asn or Lys); Xaa<sub>17</sub> =  
 (Glu, Asp or Asn); Xaa<sub>18</sub> = (Thr, Ala or Val); Xaa<sub>19</sub> =  
 (Pro or Ala); Xaa<sub>20</sub> = (Gln or Lys); Xaa<sub>21</sub> = (Asn or  
 Ser); Xaa<sub>22</sub> = (Ile or Thr); Xaa<sub>23</sub> = (Phe or Tyr);  
 Xaa<sub>24</sub> = (Asp, Glu or Ser); Xaa<sub>25</sub> = (Ser or Asn); Xaa<sub>26</sub>  
 = (Ile or Asp); Xaa<sub>27</sub> = (Lys or Arg); Xaa<sub>28</sub> = (Tyr,  
 Ala or His); and Xaa<sub>29</sub> = (Arg or Lys).

In still another preferred aspect, the invention comprises a polypeptide chain useful as a subunit of a dimeric osteogenic protein that is capable of inducing endochondral bone formation when implanted in a mammal in association with a matrix wherein the amino acid sequence of the polypeptide chain is at least 70% homologous with the minimally required amino acid sequence of mOP-2 and, preferably, at least 80% homologous with this sequence.



The identification of mOP-2 and hOP-2 represents the discovery of osteogenic proteins having a minimally required active region comprising eight cysteine residues. Thus, in another aspect, the invention comprises species of polypeptide chains having the amino acid sequence (Sequence ID No. 7):

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Cys  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa
 1      5      10
Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa
      15      20
Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Cys  Xaa  Xaa  Xaa
      25      30
Cys  Xaa  Xaa  Xaa  Xaa  Xaa  Cys  Xaa  Xaa  Xaa  Xaa
      35      40
Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa
45      50      55
Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Cys
      60      65
Cys  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa
      70      75
Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa
      80      85
Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Cys
      90      95
Xaa  Cys  Xaa.
100

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Or, alternatively, (Sequence ID No. 8):

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Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa
 1      5      10
Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa
      15      20
Xaa  Xaa  Cys  Xaa  Xaa  Xaa  Cys  Xaa  Xaa  Xaa  Xaa
      25      30
Xaa  Cys  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa
      35      40
Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa
45      50      55
Xaa  Xaa  Xaa  Xaa  Xaa  Cys  Cys  Xaa  Xaa  Xaa  Xaa
      60      65
Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa
      70      75
Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa  Xaa
      80      85
Xaa  Xaa  Xaa  Xaa  Xaa  Cys  Xaa  Cys  Xaa,
      90      95

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wherein each Xaa independently represents one of the 20 naturally occurring L-isomer,  $\alpha$ -amino acids which, together with the determined cysteine residues, define a polypeptide chain such that dimeric osteogenic proteins comprising this polypeptide chain have a conformation capable of inducing endochondral bone formation when implanted in a mammal in association with a matrix.

The invention provides recombinant dimeric proteins comprising any of the polypeptide chains described above, as well as allelic variants, and naturally-occurring or biosynthetic mutants thereof, and osteogenic devices comprising these proteins.

The novel polypeptide chains and the osteogenic proteins they comprise can be expressed from intact or truncated cDNA or from synthetic DNAs in procaryotic or eucaryotic host cells, and then purified, cleaved, refolded, dimerized, and implanted in experimental animals. Currently preferred host cells include E.coli or mammalian cells, such as CHO, COS or BSC cells. The osteogenic protein of the invention may include forms having varying glycosylation patterns, varying N-termini, a family of related proteins having regions of amino acid sequence homology, and active truncated or mutated forms of native or biosynthetic proteins, produced by expression of recombinant DNA in host cells.

Thus, in view of this disclosure, skilled genetic engineers can isolate genes from cDNA or genomic libraries of various different species which

encode appropriate amino acid sequences, or construct DNAs from oligonucleotides, and then can express them in various types of host cells, including both procaryotes and eucaryotes, to produce large quantities of active proteins capable of inducing bone formation in mammals including humans.

The osteogenic proteins are useful in clinical applications in conjunction with a suitable delivery or support system (matrix). The matrix is made up of particles of porous materials. The pores must be of a dimension to permit progenitor cell migration and subsequent differentiation and proliferation. The particle size should be within the range of 70 - 850  $\mu\text{m}$ , preferably 150 $\mu\text{m}$  - 420 $\mu\text{m}$ . It may be fabricated by close packing particulate material into a shape spanning the bone defect, or by otherwise structuring as desired a material that is biocompatible (non-inflammatory) and, biodegradable in vivo to serve as a "temporary scaffold" and substratum for recruitment of migratory progenitor cells, and as a base for their subsequent anchoring and proliferation. Currently preferred carriers include particulate, demineralized, guanidine extracted, species-specific (allogenic) bone, and specially treated particulate, protein extracted, demineralized, xenogenic bone. Optionally, such xenogenic bone powder matrices also may be treated with proteases such as trypsin and/or fibril modifying agents to increase the intraparticle intrusion volume and surface area. Useful agents include solvents such as dichloromethane, trichloroacetic acid, acetonitrile and acids such as trifluoroacetic acid and hydrogen fluoride.

Alternatively, the matrix may be treated with a hot aqueous medium having a temperature within the range of about 37°C to 65°C. Other potentially useful matrix materials comprise collagen, homopolymers and copolymers of glycolic acid and lactic acid, hydroxyapatite, tricalcium phosphate and other calcium phosphates.

The osteogenic proteins and implantable osteogenic devices enabled and disclosed herein will permit the physician to obtain optimal predictable bone formation to correct, for example, acquired and congenital craniofacial and other skeletal or dental anomalies (Glowacki et al. (1981) Lancet 1:959-963). The devices may be used to induce local endochondral bone formation in non-union fractures as demonstrated in animal tests, and in other clinical applications including periodontal applications where bone formation is required. Another potential clinical application is in cartilage repair, for example, in the treatment of osteoarthritis.

Brief Description of the Drawing

The foregoing and other objects of this invention, the various features thereof, as well as the invention itself, may be more fully understood from the following description, when read together with the accompanying drawings, in which:

FIGURE 1 compares the amino acid sequences of the mature mOP-2 and hOP-2 polypeptide chains; and

FIGURE 2 compares the amino acid sequences of the mature OP-1 (hOP-1), mOP-1, mOP-2 and hOP-2 polypeptide chains.

Description

Purification protocols first were developed which enabled isolation of the osteogenic protein present in crude protein extracts from mammalian bone. (See PCT US 89/01453, and U.S. Serial No. 179,406 filed April 8, 1988). The development of the procedure, coupled with the availability of fresh calf bone, enabled isolation of substantially pure bovine osteogenic protein (BOP). BOP was characterized significantly; its ability to induce cartilage and ultimately endochondral bone growth in cat, rabbit, and rat were demonstrated and studied; it was shown to be able to induce the full developmental cascade of bone formation previously ascribed to unknown protein or proteins in heterogeneous bone extracts. This dose dependent and highly specific activity was present whether or not the protein was glycosylated (see U.S. Serial No. 232,630 filed 8/15/88 and Sampath et al., (1990) J. Biol. Chem. 265: pp. 13198-13205). Sequence data obtained from the bovine materials suggested probe designs which were used to isolate human genes. The OP human counterpart proteins have now been expressed and extensively characterized.

These discoveries enabled preparation of DNAs encoding totally novel, non-native protein constructs which individually as homodimers and combined with other species as heterodimers are capable of producing true endochondral bone (see PCT 89/01469, filed 4/7/89 and US Serial No. 315,342, filed 2/23/89). They also permitted expression of the natural material, truncated forms, muteins,

analogs, fusion proteins, and various other variants and constructs, from cDNAs and genomic DNAs retrieved from natural sources or from synthetic DNA produced using the techniques disclosed herein and using automated, commercially available equipment. The DNAs may be expressed using well established molecular biology and recombinant DNA techniques in procaryotic or eucaryotic host cells, and may be oxidized and refolded in vitro if necessary, to produce biologically active protein.

One of the DNA sequences isolated from human genomic and cDNA libraries encoded a previously unidentified gene, referred to herein as OP-1. The protein encoded by the isolated DNA was identified originally by amino acid homology with proteins in the TGF- $\beta$  family. Consensus splice signals were found where amino acid homologies ended, designating exon-intron boundaries. Three exons were combined to obtain a functional TGF- $\beta$ -like domain containing seven cysteines. (See, for example, U.S. Serial No. 315,342 filed 2/23/80, or Ozkaynak, E. et al., (1990) EMBO. J. 9: pp. 2085-2093).

The cDNA sequence for the mature form of OP-1, including the amino acid sequence it encodes, is represented in Seq. ID No. 6. This cDNA sequence of OP-1, and the full-length cDNA sequence (encoding the "prepro" form which contains an N-terminal signal peptide sequence), as well as various truncated forms of the gene, and fused genes, have been expressed in E. coli (see, for example, U.S. Serial No. 422, 699) and numerous mammalian cells (See, for example, U.S. Serial No. 569,920), and all have been shown to have

osteogenic activity when implanted in a mammal in association with a matrix.

Given the foregoing amino acid and DNA sequence information, various DNAs can be constructed which encode at least the minimal active domain of OP-1, and various analogs thereof (including allelic variants and those containing genetically engineered mutations), as well as fusion proteins, truncated forms of the mature proteins, and similar constructs. Moreover, DNA hybridization probes can be constructed from fragments of the OP-1 DNA or designed de novo based on the OP-1 DNA or amino acid sequence. These probes then can be used to screen different genomic and cDNA libraries to identify additional osteogenic proteins.

The DNAs can be produced by those skilled in the art using well known DNA manipulation techniques involving genomic and cDNA isolation, construction of synthetic DNA from synthesized oligonucleotides, and cassette mutagenesis techniques. 15-100mer oligonucleotides may be synthesized on a Biosearch DNA Model 8600 Synthesizer, and purified by polyacrylamide gel electrophoresis (PAGE) in Tris-Borate-EDTA buffer. The DNA then may be electroeluted from the gel. Overlapping oligomers may be phosphorylated by T4 polynucleotide kinase and ligated into larger blocks which may also be purified by PAGE.

DNAs used as hybridization probes may be labelled (e.g., as with a radioisotope, by nick-translation) and used to identify clones in a



given library containing DNA to which the probe hybridizes, following techniques well known in the art. The libraries may be obtained commercially or they may be constructed de novo using conventional molecular biology techniques. Further information on DNA library construction and hybridization techniques can be found in numerous texts known to those skilled in the art. See, for example, F.M. Ausubel., ed., Current Protocols in Molecular Biology-Vol. 1, (1989). In particular, see unit 5, "Construction of Recombinant DNA Libraries" and Unit 6, "Screening of Recombinant Libraries."

The DNA from appropriately identified clones then can be isolated, subcloned (preferably into an expression vector), and sequenced. Plasmids containing sequences of interest then can be transfected into an appropriate host cell for protein expression and further characterization. The host may be a procaryotic or eucaryotic cell since the former's inability to glycosylate protein will not destroy the protein's osteogenic activity. Useful host cells include E. coli, Saccharomyces, the insect/baculovirus cell system, myeloma cells, and various mammalian cells. The vector additionally may encode various sequences to promote correct expression of the recombinant protein, including transcription promoter and termination sequences, enhancer sequences, preferred ribosome binding site sequences, preferred mRNA leader sequences, preferred signal sequences for protein secretion, and the like. The DNA sequence encoding the gene of interest also may be manipulated to remove potentially inhibiting sequences or to minimize unwanted

secondary structure formation. The recombinant osteogenic protein also may be expressed as a fusion protein. After being translated, the protein may be purified from the cells themselves or recovered from the culture medium. All biologically active protein forms comprise dimeric species joined by disulfide bonds or otherwise associated, produced by oxidizing and refolding one or more of the various recombinant polypeptide chains within an appropriate eucaryotic cell or in vitro after expression of individual subunits. A detailed description of osteogenic protein expressed from recombinant DNA in E. coli is disclosed in U.S. Serial No. 422,699, the disclosure of which has been incorporated by reference, supra. A detailed description of osteogenic protein expressed from recombinant DNA in numerous different mammalian cells is disclosed in U.S. Serial No. 569,920, the disclosure of which is hereby incorporated by reference.

#### Exemplification

In an effort to identify additional DNA sequences encoding osteogenic proteins, a hybridization probe specific to the C-terminus of the DNA of mature OP-1 was prepared using a StuI-EcoRI digest fragment of OP-1 (base pairs 109-431 in Sequence ID No. 6), and labelled with  $^{32}\text{P}$  by nick translation, as described in the art. The C-terminus of the protein is known to encode a key functional domain ("active region") of the mature protein (amino acid residues 38-139 of Sequence ID No. 6). The C-terminus also is the region of the protein whose amino acid sequence has particular homology with

proteins in the TGF- $\beta$  super-family of regulatory proteins.

Approximately  $7 \times 10^5$  phages of an oligo(dT) primed 17.5 days p.c. mouse embryo 5' stretch cDNA (gt10) library (Clontech, Inc., Palo Alto, CA) was screened with the labelled probe. The screen was performed as described in the art, at 50°C, and in high stringency buffer (1 X SSPE, 0.1% SDS).

Five recombinant phages were purified over three rounds of screening. Phage DNA was prepared from all five phages, subjected to an EcoRI digest, subcloned into the EcoRI site of a common pUC-type plasmid modified to allow single strand sequencing, and sequenced using means well known in the art.

Two different DNAs were identified by this procedure. One DNA, referred to herein as mOP-1, has substantial homology to the mature form of OP-1 (about 98%), and is described in detail in copending (Attorney docket # CRP-001CP5). A second DNA, encoding the C-terminus of a related gene and referred to herein as mOP-2, also was identified by this procedure. The N-terminus of the gene encoding mOP-2 was identified subsequently by screening a second mouse cDNA library (Mouse PCC4 cDNA (ZAP) library, Stratagene, Inc., La Jolla, CA).

Mouse OP-2 (mOP-2) has significant homology with the minimally required C-terminal active region of OP-1 (about 74%), and less homology with the intact mature form (about 58%). The cDNA sequence, and the encoded amino acid sequence, for the full

length mOP-2 protein is depicted in Sequence ID No. 2. The full-length form of the protein is referred to as the prepro form of mOP-2, and includes a signal peptide sequence at its N-terminus. The amino acid sequence Leu-Ala-Leu-Cys-Ala-Leu (amino acid residues 13-18 of Sequence ID No. 2) is believed to constitute the cleavage site for the removal of the signal peptide sequence, leaving an intermediate form of the protein, the "pro" form, to be secreted from the expressing cell. The amino acid sequence Arg-Ala-Pro-Arg-Ala (amino acid residues 255-259 of Sequence ID No. 2) is believed to constitute the cleavage site that produces the mature form of the protein, whose sequence is depicted below (Sequence ID No. 1):

mOP-2

Ala	Ala	Arg	Pro	Leu	Lys	Arg	Arg	Gln
1				5				
Pro	Lys	Lys	Thr	Asn	Glu	Leu	Pro	His
10					15			
Pro	Asn	Lys	Leu	Pro	Gly	Ile	Phe	Asp
	20					25		
Asp	Gly	His	Gly	Ser	Arg	Gly	Arg	Glu
		30					35	
Val	Cys	Arg	Arg	His	Glu	Leu	Tyr	Val
			40					45
Arg	Phe	Arg	Asp	Leu	Gly	Trp	Leu	Asp
				50				
Trp	Val	Ile	Ala	Pro	Gln	Gly	Tyr	Ser
55					60			

Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ala
	65					70		
Phe	Pro	Leu	Asp	Ser	Cys	Met	Asn	Ala
		75					80	
Thr	Asn	His	Ala	Ile	Leu	Gln	Ser	Leu
			85					90
Val	His	Leu	Met	Lys	Pro	Asp	Val	Val
				95				
Pro	Lys	Ala	Cys	Cys	Ala	Pro	Thr	Lys
100					105			
Leu	Ser	Ala	Thr	Ser	Val	Leu	Tyr	Tyr
	110					115		
Asp	Ser	Ser	Asn	Asn	Val	Ile	Leu	Arg
		120					125	
Lys	His	Arg	Asn	Met	Val	Val	Lys	Ala
			130					135
Cys	Gly	Cys	His.					

Using a probe prepared from the pro region of mOP-2 (an EcoRI-BamHI digest fragment, bp 471-776 of Sequence ID No. 2), a human hippocampus library was screened (human hippocampus cDNA lambda (ZAP II library Stratagene, Inc., La Jolla, CA) following essentially the same procedure as for the mouse library screens. The procedure identified the N-terminus of a novel DNA encoding an amino acid sequence having substantial homology with mOP-2. The C-terminus of the gene subsequently was identified by probing a human genomic library (in lambda phage EMBL-3, Clonetech, Inc., Palo Alto, CA) with a labelled fragment from the novel human DNA in hand. The novel polypeptide chain encoded by this DNA is referred to herein as hOP-2, and has about 92% sequence homology with the mature mOP-2 (about 95%

homology within the minimally required C-terminal active region).

The cDNA sequence, and the encoded amino acid sequence, for the prepro form of hOP-2 is depicted in Sequence ID No. 4. This full-length form of the protein also includes a signal peptide sequence at its N-terminus. The amino acid sequence Leu-Ala-Leu-Cys-Ala-Leu (amino acid residues 13-18 of Sequence ID No. 4) is believed to constitute the cleavage site for the removal of the signal peptide sequence, leaving an intermediate form of the protein, the "pro" form, to be secreted from the expressing cell. The amino acid sequence Arg-Thr-Pro-Arg-Ala (amino acid residues 257-261 of Sequence ID No. 4) is believed to constitute the cleavage site that produces what is believed to be the mature form of the protein, whose sequence is depicted below (Sequence ID No. 3):

hOP-2

Ala	Val	Arg	Pro	Leu	Arg	Arg	Arg	Gln
1				5				
Pro	Lys	Lys	Ser	Asn	Glu	Leu	Pro	Gln
10					15			
Ala	Asn	Arg	Leu	Pro	Gly	Ile	Phe	Asp
	20					25		
Asp	Val	Asn	Gly	Ser	His	Gly	Arg	Gln
		30					35	
Val	Cys	Arg	Arg	His	Glu	Leu	Tyr	Val
			40					45
Ser	Phe	Gln	Asp	Leu	Gly	Trp	Leu	Asp
				50				

Tyr 55	Val	Ile	Ala	Pro	Gln 60	Gly	Tyr	Ser
Ala	Tyr 65	Tyr	Cys	Glu	Gly	Glu 70	Cys	Ser
Phe	Pro	Leu 75	Asp	Ser	Cys	Met	Asn 80	Ala
Thr	Asn	His	Ala 85	Ile	Leu	Gln	Ser	Leu 90
Val	His	Leu	Met	Lys 95	Pro	Asn	Ala	Val
Pro 100	Lys	Ala	Cys	Cys	Ala 105	Pro	Thr	Lys
Leu 110	Ser	Ala	Thr	Ser	Val	Leu 115	Tyr	Tyr
Asp	Glu	Ser 120	Asn	Asn	Val	Ile	Leu 125	Arg
Lys	Ala	Arg	Asn 130	Met	Val	Val	Lys	Ala 135
Cys	Gly	Cys	His.					

Additional mature species thought to be active include two short sequences:

hOP-2P (SEQ ID NO. 9)

			Pro 1	Leu	Arg	Arg	Arg 5	Gln
Pro	Lys	Lys	Ser 10	Asn	Glu	Leu	Pro	Gln 15
Ala	Asn	Arg	Leu	Pro 20	Gly	Ile	Phe	Asp
Asp 25	Val	Asn	Gly	Ser	His 30	Gly	Arg	Gln

Val	Cys 35	Arg	Arg	His	Glu	Leu 40	Tyr	Val
Ser	Phe	Gln 45	Asp	Leu	Gly	Trp	Leu 50	Asp
Tyr	Val	Ile	Ala 55	Pro	Gln	Gly	Tyr	Ser 60
Ala	Tyr	Tyr	Cys	Glu 65	Gly	Glu	Cys	Ser
Phe 70	Pro	Leu	Asp	Ser	Cys 75	Met	Asn	Ala
Thr 80	Asn	His	Ala	Ile	Leu	Gln 85	Ser	Leu
Val	His	Leu 90	Met	Lys	Pro	Asn	Ala 95	Val
Pro	Lys	Ala	Cys 100	Cys	Ala	Pro	Thr	Lys 105
Leu	Ser	Ala	Thr	Ser 110	Val	Leu	Tyr	Tyr
Asp 115	Glu	Ser	Asn	Asn	Val 120	Ile	Leu	Arg
Lys	Ala 125	Arg	Asn	Met	Val	Val 130	Lys	Ala
Cys	Gly	Cys 135	His;					

HOP-2R (SEQ ID NO. 10):

						Arg 1	Arg	Gln
Pro	Lys 5	Lys	Ser	Asn	Glu	Leu 10	Pro	Gln
Ala	Asn	Arg 15	Leu	Pro	Gly	Ile	Phe 20	Asp



Asp	Val	Asn	Gly	Ser	His	Gly	Arg	Gln
			25					30
Val	Cys	Arg	Arg	His	Glu	Leu	Tyr	Val
			35					
Ser	Phe	Gln	Asp	Leu	Gly	Trp	Leu	Asp
40					45			
Tyr	Val	Ile	Ala	Pro	Gln	Gly	Tyr	Ser
	50					55		
Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ser
		60					65	
Phe	Pro	Leu	Asp	Ser	Cys	Met	Asn	Ala
			70					75
Thr	Asn	His	Ala	Ile	Leu	Gln	Ser	Leu
			80					
Val	His	Leu	Met	Lys	Pro	Asn	Ala	Val
85					90			
Pro	Lys	Ala	Cys	Cys	Ala	Pro	Thr	Lys
	95					100		
Leu	Ser	Ala	Thr	Ser	Val	Leu	Tyr	Tyr
		105					110	
Asp	Glu	Ser	Asn	Asn	Val	Ile	Leu	Arg
			115					120
Lys	Ala	Arg	Asn	Met	Val	Val	Lys	Ala
				125				
Cys	Gly	Cys	His;					
130								

and a longer sequence:

hOP-2S (SEQ ID NO. 11)

					Ser	Gln	Gln
					1		
Pro	Phe	Val	Val	Thr	Phe	Arg	Ala
	5				10		

Ser	Pro	Ser 15	Pro	Ile	Arg	Thr	Pro 20	Arg
Ala	Val	Arg	Pro 25	Leu	Arg	Arg	Arg	Gln 30
Pro	Lys	Lys	Ser	Asn 35	Glu	Leu	Pro	Gln
Ala 40	Asn	Arg	Leu	Pro	Gly 45	Ile	Phe	Asp
Asp	Val 50	Asn	Gly	Ser	His	Gly 55	Arg	Gln
Val	Cys	Arg 60	Arg	His	Glu	Leu	Tyr 65	Val
Ser	Phe	Gln	Asp 70	Leu	Gly	Trp	Leu	Asp 75
Tyr	Val	Ile	Ala	Pro 80	Gln	Gly	Tyr	Ser
Ala 85	Tyr	Tyr	Cys	Glu	Gly 90	Glu	Cys	Ser
Phe	Pro 95	Leu	Asp	Ser	Cys	Met 100	Asn	Ala
Thr	Asn	His 105	Ala	Ile	Leu	Gln	Ser 110	Leu
Val	His	Leu	Met 115	Lys	Pro	Asn	Ala	Val 120
Pro	Lys	Ala	Cys	Cys 125	Ala	Pro	Thr	Lys
Leu 130	Ser	Ala	Thr	Ser	Val 135	Leu	Tyr	Tyr
Asp	Glu 140	Ser	Asn	Asn	Val	Ile 145	Leu	Arg
Lys	Ala	Arg 150	Asn	Met	Val	Val	Lys 165	Ala
Cys	Gly	Cys	His. 170					

It should be noted that the nucleic acid sequence encoding the N-terminus of the pre-pro form of both mOP2 and hOP2 is rich in guanidine and cytosine base pairs. As will be appreciated by those skilled in the art, sequencing such a "G-C rich" region can be problematic, due to stutter and/or band compression. Accordingly, the possibility of sequencing errors in this region can not be ruled out. However, the definitive amino acid sequence can be determined readily by expressing the protein from recombinant DNA using, for example, any of the means disclosed herein, and sequencing the polypeptide chain by conventional peptide sequencing methods well known in the art.

Figure 1 compares the amino acid sequences of mature mOP2 and hOP2. As is evident from the figure, the sequence homology between the mature forms of these two proteins is substantial. The two sequences differ at only 17 positions. Identity is indicated by three dots (...) in the mOP-2 sequence.

Figure 2 compares the sequences for the mature form of all four OP proteins. The greatest homology between sequences (73%, indicated by dots) occurs within the minimally required active region (residues 38-139). The high degree of homology within this region is not surprising, as the mature C-terminal domains of TGF- $\beta$ -like proteins generally are found to be highly conserved across different animal species. The OP proteins have less sequence homology with CBMP2a and CBMP2b, two other osteogenic proteins described in U.S. 3,153,342 and 4,833,699. Like OP-1 and mOP-1, the mature CBMP protein sequences

have seven cysteine residues. OP-1 (hOP-1) and mOP-1 have about 64% sequence homology with the CBMP sequences within the minimally required C-terminal active region. Mouse OP-2 (mOP-2) and hOP-2 have only about 58% homology with the CBMP sequences in this region.

A preferred generic amino acid sequence useful as a subunit of a dimeric osteogenic protein capable of inducing endochondral bone or cartilage formation when implanted in a mammal in association with a matrix, and which incorporates the maximum homology between the four OP proteins, is described below (sequence ID No. 5):

Cys	Xaa <sub>1</sub>	Xaa <sub>2</sub>	His	Glu	Leu	Tyr	Val	Xaa <sub>3</sub>	Phe	
1					5					10
Xaa <sub>4</sub>	Asp	Leu	Gly	Trp	Xaa <sub>5</sub>	Asp	Trp	Xaa <sub>6</sub>	Ile	
				15						20
Ala	Pro	Xaa <sub>7</sub>	Gly	Tyr	Xaa <sub>8</sub>	Ala	Tyr	Tyr	Cys	
				25						30
Glu	Gly	Cys	Xaa <sub>9</sub>	Phe	Pro	Leu	Xaa <sub>10</sub>	Ser	Xaa <sub>11</sub>	
				35						40
Met	Asn	Ala	Thr	Asn	His	Ala	Ile	Xaa <sub>12</sub>	Thr	
				45						50
Leu	Xaa <sub>13</sub>	Xaa <sub>14</sub>	Xaa <sub>15</sub>	Xaa <sub>16</sub>	Xaa <sub>17</sub>	Xaa <sub>18</sub>	Val			
				55						
Pro	Lys	Xaa <sub>19</sub>	Cys	Cys	Ala	Pro	Thr	Xaa <sub>20</sub>	Leu	
	60							65		
Xaa <sub>21</sub>	Ala	Xaa <sub>22</sub>	Ser	Val	Leu	Tyr	Xaa <sub>23</sub>	Asp		
	70						75			
Xaa <sub>24</sub>	Ser	Xaa <sub>25</sub>	Asn	Val	Xaa <sub>26</sub>	Leu	Xaa <sub>27</sub>	Lys		
		80						85		

Xaa<sub>28</sub> Pro Asn Met Val Val Xaa<sub>29</sub> Ala Cys Gly  
90 95

Cys His,

wherein Xaa<sub>1</sub> = (Lys or Arg); Xaa<sub>2</sub> = (Lys or Arg);  
Xaa<sub>3</sub> = (Ser or Arg); Xaa<sub>4</sub> = (Arg or Gln); Xaa<sub>5</sub> = (Gln  
or Leu); Xaa<sub>6</sub> = (Ile or Val); Xaa<sub>7</sub> = (Glu or Gln);  
Xaa<sub>8</sub> = (Ala or Ser); Xaa<sub>9</sub> = (Ala or Ser); Xaa<sub>10</sub> =  
(Asn or Asp); Xaa<sub>11</sub> = (Tyr or Cys); Xaa<sub>12</sub> = (Val or  
Leu); Xaa<sub>13</sub> = (His or Asn); Xaa<sub>14</sub> = (Phe or Leu);  
Xaa<sub>15</sub> = (Ile or Met); Xaa<sub>16</sub> = (Asn or Lys); Xaa<sub>17</sub> =  
(Glu, Asp or Asn); Xaa<sub>18</sub> = (Thr, Ala or Val); Xaa<sub>19</sub> =  
(Pro or Ala); Xaa<sub>20</sub> = (Gln or Lys); Xaa<sub>21</sub> = (Asn or  
Ser); Xaa<sub>22</sub> = (Ile or Thr); Xaa<sub>23</sub> = (Phe or Tyr);  
Xaa<sub>24</sub> = (Asp, Glu or Ser); Xaa<sub>25</sub> = (Ser or Asn); Xaa<sub>26</sub>  
= (Ile or Asp); Xaa<sub>27</sub> = (Lys or Arg); Xaa<sub>28</sub> = (Tyr,  
Ala or His); and Xaa<sub>29</sub> = (Arg or Lys).

The high degree of homology exhibited between the various OP proteins suggests that the novel OP proteins identified herein will purify essentially as OP-1 does, or with only minor modifications of the protocols disclosed for OP-1. Similarly, purified mOP-1, mOP-2, and hOP-2 are predicted to have an apparent molecular weight of about 18 kD as reduced single subunits, and an apparent molecular weight of about 36 kD as oxidized dimers, as determined by comparison with molecular weight standards on an SDS-polyacrylamide electrophoresis gel. Unglycosylated dimers (e.g., one expressed from *E. coli*) are predicted to have a molecular weight of about 27 kD. There appears to be one potential N glycosylation sites in the mature forms of mOP-2 and hOP-2.

The identification of osteogenic proteins having an active domain comprising eight cysteine residues also allows one to construct osteogenic polypeptide chains patterned after either of the following template amino acid sequences (Sequence ID. No. 7 or 8), or to identify additional osteogenic proteins having this sequence, wherein each Xaa independently represents one of the 20 naturally-occurring L-isomer,  $\alpha$ -amino acids. Biosynthetic constructs patterned after this template readily are constructed using conventional DNA synthesis or peptide synthesis techniques well known in the art. Once constructed, osteogenic proteins comprising these polypeptide chains can be tested as disclosed herein. Sequence ID No. 7:

```

Cys1 Xaa Xaa Xaa Xaa5 Xaa Xaa Xaa Xaa Xaa10 Xaa
Xaa Xaa Xaa Xaa15 Xaa Xaa Xaa Xaa Xaa20 Xaa Xaa
Xaa Xaa Xaa25 Xaa Xaa Xaa Xaa Cys30 Xaa Xaa Xaa
Cys35 Xaa35 Xaa Xaa Xaa Xaa Cys40 Xaa Xaa Xaa Xaa
Xaa45 Xaa Xaa Xaa Xaa Xaa50 Xaa Xaa Xaa Xaa Xaa55
Xaa Xaa Xaa Xaa Xaa60 Xaa Xaa Xaa Xaa Xaa65 Cys
Cys Xaa Xaa Xaa70 Xaa Xaa Xaa Xaa Xaa75 Xaa Xaa
Xaa Xaa Xaa80 Xaa Xaa Xaa Xaa Xaa85 Xaa Xaa Xaa
Xaa Xaa90 Xaa Xaa Xaa Xaa Xaa95 Xaa Xaa Xaa Cys
Xaa100 Cys Xaa.

```

Or, alternatively, (Sequence ID No. 8):

```

Xaa1 Xaa Xaa Xaa Xaa5 Xaa Xaa Xaa Xaa Xaa10 Xaa

```

Xaa	Xaa	Xaa	Xaa <sub>15</sub>	Xaa	Xaa	Xaa	Xaa	Xaa <sub>20</sub>	Xaa	Xaa
Xaa	Xaa	Cys <sub>25</sub>	Xaa	Xaa	Xaa	Cys	Xaa <sub>30</sub>	Xaa	Xaa	Xaa
Xaa	Cys <sub>35</sub>	Xaa	Xaa	Xaa	Xaa	Xaa <sub>40</sub>	Xaa	Xaa	Xaa	Xaa
Xaa <sub>45</sub>	Xaa	Xaa	Xaa	Xaa	Xaa <sub>50</sub>	Xaa	Xaa	Xaa	Xaa	Xaa <sub>55</sub>
Xaa	Xaa	Xaa	Xaa	Xaa <sub>60</sub>	Cys	Cys	Xaa	Xaa	Xaa <sub>65</sub>	Xaa
Xaa	Xaa	Xaa	Xaa <sub>70</sub>	Xaa	Xaa	Xaa	Xaa	Xaa <sub>75</sub>	Xaa	Xaa
Xaa	Xaa	Xaa <sub>80</sub>	Xaa	Xaa	Xaa	Xaa	Xaa <sub>85</sub>	Xaa	Xaa	Xaa
Xaa	Xaa <sub>90</sub>	Xaa	Xaa	Xaa	Cys	Xaa <sub>95</sub>	Cys	Xaa		

#### MATRIX PREPARATION

Practice of the invention as part of an osteogenic ddevice capable of inducing endochondral bone formation in a mammal requires the availability of bone as a carrier material. The currently preferred bone is mammalian bone, e.g., bovine. The bone is cleaned, demarrowed, delipidated, demineralized, reduced to particles of an appropriate size, extracted to remove soluble proteins, sterilized, and otherwise treated as disclosed herein to produce an implantable material useful in a variety of clinical settings.

Matrices of various shapes fabricated from the material of the invention may be implanted surgically for various purposes. Chief among these is to serve as a matrix for bone formation in various orthopedic, periodontal, and reconstructive procedures, as a sustained release carrier, or as a collagenous coating for implants. The matrix may be shaped as desired in anticipation of surgery or

shaped by the physician or technician during surgery. Thus, the material may be used for topical, subcutaneous, intraperitoneal, or intramuscular implants; it may be shaped to span a nonunion fracture or to fill a bone defect. In bone formation or conduction procedures, the material is slowly absorbed by the body and is replaced by bone in the shape of or very nearly the shape of the implant.

Various growth factors, hormones, enzymes, therapeutic compositions, antibiotics, and other body treating agents also may be sorbed onto the carrier material and will be released over time when implanted as the matrix material is slowly absorbed. Thus, various known growth factors such as EGF, PDGF, IGF, FGF, TGF- $\alpha$ , and TGF- $\beta$  may be released in vivo. The material can be used to release chemotherapeutic agents, insulin, enzymes, or enzyme inhibitors.

Details of how to make and how to use the materials of the invention are disclosed below.

1. Preparation of Demineralized Bone

Demineralized bovine bone matrix is prepared by previously published procedures (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591-6595). Bovine diaphyseal bones (age 1-10 days) are obtained from a local slaughterhouse and used fresh. The bones are stripped of muscle and fat, cleaned of periosteum, demarrowed by pressure with cold water, dipped in cold absolute ethanol, and stored at -20°C. They are then dried and fragmented by



crushing and pulverized in a large mill. Care is taken to prevent heating by using liquid nitrogen. The pulverized bone is milled to a particle size in the range of 70-850  $\mu\text{m}$ , preferably 150  $\mu\text{m}$ -420  $\mu\text{m}$ , and is defatted by two washes of approximately two hours duration with three volumes of chloroform and methanol (3:1). The particulate bone is then washed with one volume of absolute ethanol and dried over one volume of anhydrous ether yielding defatted bone powder. The defatted bone powder is then demineralized by four successive treatments with 10 volumes of 0.5 N HCl at 4°C for 40 min. Finally, neutralizing washes are done on the demineralized bone powder with a large volume of water.

2. Guanidine Extraction

Demineralized bone matrix thus prepared is extracted with 5 volumes of 4 M guanidine-HCl, 50mM Tris-HCl, pH 7.0 for 16 hr. at 4°C. The suspension is filtered. The insoluble material is collected and used to fabricate the matrix. The material is mostly collagenous in nature. It is devoid of osteogenic or chondrogenic activity.

3. Matrix Treatments

The major component of all bone matrices is Type-I collagen. In addition to collagen, demineralized bone extracted as disclosed above includes non-collagenous proteins which may account for 5% of its mass. In a xenogenic matrix, these noncollagenous components may present themselves as potent antigens, and may constitute immunogenic

and/or inhibitory components. These components also may inhibit osteogenesis in allogenic implants by interfering with the developmental cascade of bone differentiation. It has been discovered that treatment of the matrix particles with a collagen fibril-modifying agent extracts potentially unwanted components from the matrix, and alters the surface structure of the matrix material. Useful agents include acids, organic solvents or heated aqueous media. Various treatments are described below. A detailed physical analysis of the effect these fibril-modifying agents have on demineralized, guanidine-extracted bone collagen particles is disclosed in copending U.S. Patent Application Serial No. 483,913, filed February 22, 1990.

After contact with the fibril-modifying agent, the treated matrix is washed to remove any extracted components, following a form of the procedure set forth below:

1. Suspend in TBS (Tris-buffered saline) 1g/200 ml and stir at 4°C for 2 hrs; or in 6 M urea, 50 mM Tris-HCl, 500 mM NaCl, pH 7.0 (UTBS) or water and stir at room temperature (RT) for 30 minutes (sufficient time to neutralize the pH);
2. Centrifuge and repeat wash step; and
3. Centrifuge; discard supernatant; water wash residue; and then lyophilize.

#### Acid Treatments

1. Trifluoroacetic acid.

Trifluoroacetic acid is a strong non-oxidizing acid that is a known swelling agent for proteins, and which modifies collagen fibrils.

Bovine bone residue prepared as described above is sieved, and particles of the appropriate size are collected. These particles are extracted with various percentages (1.0% to 100%) of trifluoroacetic acid and water (v/v) at 0°C or room temperature for 1-2 hours with constant stirring. The treated matrix is filtered, lyophilized, or washed with water/salt and then lyophilized.

2. Hydrogen Fluoride.

Like trifluoroacetic acid, hydrogen fluoride is a strong acid and swelling agent, and also is capable of altering intraparticle surface structure. Hydrogen fluoride is also a known deglycosylating agent. As such, HF may function to increase the osteogenic activity of these matrices by removing the antigenic carbohydrate content of any glycoproteins still associated with the matrix after guanidine extraction.

Bovine bone residue prepared as described above is sieved, and particles of the appropriate size are collected. The sample is dried in vacuo over P<sub>2</sub>O<sub>5</sub>, transferred to the reaction vessel and exposed to anhydrous hydrogen fluoride (10-20 ml/g of matrix) by distillation onto the sample at -70°C. The vessel is allowed to warm to 0°C and the reaction

mixture is stirred at this temperature for 120 minutes. After evaporation of the hydrogen fluoride in vacuo, the residue is dried thoroughly in vacuo over KOH pellets to remove any remaining traces of acid. Extent of deglycosylation can be determined from carbohydrate analysis of matrix sample taken before and after treatment with hydrogen fluoride, after washing the samples appropriately to remove non-covalently bound carbohydrates. SDS-extracted protein from HF-treated material is negative for carbohydrate as determined by CON A blotting.

The deglycosylated bone matrix is next washed twice in TBS (Tris-buffered saline) or UTBS, water-washed, and then lyophilized.

Other acid treatments are envisioned in addition to HF and TFA. TFA is a currently preferred acidifying reagent in these treatments because of its volatility. However, it is understood that other, potentially less caustic acids may be used, such as acetic or formic acid.

#### Solvent Treatment

1. Dichloromethane.

Dichloromethane (DCM) is an organic solvent capable of denaturing proteins without affecting their primary structure. This swelling agent is a common reagent in automated peptide synthesis, and is used in washing steps to remove components.

Bovine bone residue, prepared as described above, is sieved, and particles of the appropriate size are incubated in 100% DCM or, preferably, 99.9% DCM/0.1% TFA. The matrix is incubated with the swelling agent for one or two hours at 0°C or at room temperature. Alternatively, the matrix is treated with the agent many times (X3) with short washes (20 minutes each) with no incubation.

## 2. Acetonitrile.

Acetonitrile (ACN) is an organic solvent, capable of denaturing proteins without affecting their primary structure. It is a common reagent used in high-performance liquid chromatography, and is used to elute proteins from silica-based columns by perturbing hydrophobic interactions.

Bovine bone residue particles of the appropriate size, prepared as described above, are treated with 100% ACN (1.0 g/30 ml) or, preferably, 99.9% ACN/0.1% TFA at room temperature for 1-2 hours with constant stirring. The treated matrix is then water-washed, or washed with urea buffer, or 4 M NaCl and lyophilized. Alternatively, the ACN or ACN/TFA treated matrix may be lyophilized without wash.

## 3. Isopropanol.

Isopropanol is also an organic solvent capable of denaturing proteins without affecting their primary structure. It is a common reagent used to elute proteins from silica HPLC columns.

Bovine bone residue particles of the appropriate size prepared as described above are treated with 100% isopropanol (1.0 g/30 ml) or, preferably, in the presence of 0.1% TFA, at room temperature for 1-2 hours with constant stirring. The matrix is then water-washed or washed with urea buffer or 4 M NaCl before being lyophilized.

#### 4. Chloroform

Chloroform also may be used to increase surface area of bone matrix like the reagents set forth above, either alone or acidified.

Treatment as set forth above is effective to assure that the material is free of pathogens prior to implantation.

#### Heat Treatment

Various amounts of delipidated, demineralized guanidine-extracted bone collagen are heated in water (1g/30ml) under constant stirring in a glass flask, water jacketed, and maintained at a given temperature for 1 hour. The water also may be replaced with 0.1M acetic acid to help "swell" the collagen before heating. The temperature employed is held constant at a temperature within the range of about 37°C to 75°C. After the heat treatment, the matrix is filtered and lyophilized and used for implant. The currently preferred heat treatment temperature is within the range of 45°C to 60°C.

The collagen matrix used as a carrier material for the osteogenic proteins of this invention preferably takes the form of a fine powder, insoluble in water, comprising nonadherent particles. It may be used simply by packing into the volume where new bone growth or sustained release is desired, held in place by surrounding tissue. Alternatively, the powder may be encapsulated in, e.g., a gelatin or polylactic acid coating, which is adsorbed readily by the body. The powder may be shaped to a volume of given dimensions and held in that shape by interadhering the particles using, for example, soluble, species biocompatible collagen. The material may also be produced in sheet, rod, bead, or other macroscopic shapes.

#### FABRICATION OF OSTEOGENIC DEVICE

The osteogenic protein disclosed herein, and other constructs, can be combined and dispersed in a suitable matrix preparation using any of the methods described below:

##### 1. Ethanol Precipitation

Matrix is added to osteogenic protein dissolved in guanidine-HCl. Samples are vortexed and incubated at a low temperature. Samples are then further vortexed. Cold absolute ethanol is added to the mixture which is then stirred and incubated. After centrifugation (microfuge, high speed) the supernatant is discarded. The matrix is washed with cold concentrated ethanol in water and then lyophilized.

2. Acetonitrile Trifluoroacetic  
Acid Lyophilization

In this procedure, osteogenic protein in an acetonitrile trifluoroacetic acid (ACN/TFA) solution was added to the carrier material. Samples were vigorously vortexed many times and then lyophilized. Osteogenic protein was added in varying concentrations, and at several levels of purity. This method is currently preferred.

3. Urea Lyophilization

For those osteogenic proteins that are prepared in urea buffer, the protein is mixed with the matrix material, vortexed many times, and then lyophilized. The lyophilized material may be used "as is" for implants.

4. Buffered Saline Lyophilization

OP preparations in physiological saline may also be vortexed with the matrix and lyophilized to produce osteogenically active material.

These procedures also can be used to adsorb other active therapeutic drugs, hormones, and various bioactive species for sustained release purposes.

BIOASSAY

The functioning of the various matrices can be evaluated with an in vivo rat bioassay. Studies in rats show the osteogenic effect in an appropriate



matrix to be dependent on the dose of osteogenic protein dispersed in the matrix. No activity is observed if the matrix is implanted alone. Demineralized, guanidine extracted xenogenic bone matrix materials of the type described in the literature are ineffective as a carrier, fail to induce bone, and produce an inflammatory and immunological response when implanted unless treated as disclosed above. Many of the allogenic matrix materials also are ineffective as carriers. The following sets forth various procedures for preparing osteogenic devices from appropriately prepared osteogenic proteins and matrix materials prepared as set forth above, and for evaluating their osteogenic utility.

#### Implantation

The bioassay for bone induction as described by Sampath and Reddi (Proc. Natl. Acad. Sci. USA (1983) 80: 6591-6595), herein incorporated by reference, may be used to monitor endochondral bone differentiation activity. This assay consists of implanting the bovine test samples xenogenically in subcutaneous sites in recipient rats under ether anesthesia. Male Long-Evans rats, aged 28-32 days, were used. A vertical incision (1 cm) is made under sterile conditions in the skin over the thoracic region, and a pocket is prepared by blunt dissection. Approximately 25 mg of the test sample is implanted deep into the pocket and the incision is closed with a metallic skin clip. The day of implantation is designated as day one of the experiment. Implants are generally removed on day

12. The heterotropic site allows for the study of bone induction without the possible ambiguities resulting from the use of orthotropic sites.

#### Cellular Events

Successful implants exhibit a controlled progression through the stages of matrix induced endochondral bone development including: (1) transient infiltration by polymorphonuclear leukocytes on day one; (2) mesenchymal cell migration and proliferation on days two and three; (3) chondrocyte appearance on days five and six; (4) cartilage matrix formation on day seven; (5) cartilage calcification on day eight; (6) vascular invasion, appearance of osteoblasts, and formation of new bone on days nine and ten; (7) appearance of osteoblastic and bone remodeling and dissolution of the implanted matrix on days twelve to eighteen; and (8) hematopoietic bone marrow differentiation in the ossicle on day twenty-one. The shape of the new bone conforms to the shape of the implanted matrix.

#### Histological Evaluation

Histological sectioning and staining is preferred to determine the extent of osteogenesis in the implants. Implants are fixed in Bouins Solution, embedded in paraffin, and cut into 6-8  $\mu$ m sections. Staining with toluidine blue or hemotoxylin/eosin demonstrates clearly the ultimate development of endochondral bone. Twelve day implants are usually sufficient to determine whether the implants contain newly induced bone.

### Biological Markers

Alkaline phosphatase activity may be used as a marker for osteogenesis. The enzyme activity may be determined spectrophotometrically after homogenization of the implant. The activity peaks at 9-10 days in vivo and thereafter slowly declines. Implants showing no bone development by histology have little or no alkaline phosphatase activity under these assay conditions. The assay is useful for quantitation and obtaining an estimate of bone formation quickly after the implants are removed from the rat. Alternatively, the amount of bone formation can be determined by measuring the calcium content of the implant.

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.